

**EFFECT OF LYSINE MODIFICATION ON THE
CONFORMATIONAL STABILITY OF α -AMYLASE**

TAN CHEAU YUAAN

**DISSERTATION SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF MASTER OF
SCIENCE**

**INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR**

2011

ABSTRACT

Chemical modifications of *Bacillus licheniformis* α -amylase (BLA) were carried out using 100 molar excess of succinic anhydride over protein, 0.66 M potassium cyanate and 0.46 M O-methylisourea under standard conditions to prepare different modified derivatives. The percentage of modification as determined by TNBSA reaction method was found to be 42% for succinylated (S₄₂), 81% for carbamylated (C₈₁) and 88% for guanidinated (G₈₈) BLA derivatives. These modified preparations were checked for their size and charge homogeneity by gel chromatography on Sephacryl S-200 HR column and polyacrylamide gel electrophoresis. Conformational changes in these preparations were detected by analytical gel chromatography on a calibrated Sephacryl S-200 HR column by determining the Stokes radii of native and modified derivatives. C₈₁ BLA was found to have the highest Stokes radius value followed by S₄₂ and G₈₈ BLAs. Conformational stabilities of native and modified BLA derivatives were investigated by urea denaturation using circular dichroism (CD) spectroscopy. Denaturation profiles showed a decrease in the stability of modified BLAs being more pronounced in C₈₁ BLA. In addition, the effect of calcium on urea and guanidine hydrochloride (GdnHCl) denaturation of native and calcium-depleted enzymes was also analyzed by CD, fluorescence spectroscopy and biological activity. Based on these results, calcium was found to stabilize the enzyme against both urea and GdnHCl denaturations.

ABSTRAK

Modifikasi kimia digunakan untuk *Bacillus licheniformis* α -amilase (BLA) dengan menggunakan 100 molar ekseks suksinik anhidrida melebihi protein, 0.66 M kalium sianat dan 0.46 M O-metilisourea dalam kondisi tetap untuk menyediakan pelbagai derivatif modifikasi. Peratus modifikasi ditentukan melalui tindak balas protein dengan TNBSA dan ditetapkan sebagai 42% untuk suksinilasi (S₄₂), 81% untuk karbamilasi (C₈₁) dan 88% untuk guanidinasi (G₈₈) BLA. Ketulenan dari segi saiz dan cas untuk semua protein yang telah melalui process modifikasi ditentukan dengan kaedah gel kromatografi dengan menggunakan kolum Sephacryl S-200 HR dan poliakrilamida gel electrophoresis. Perubahan konformasi dalam penyediaan tersebut pula ditentukan dengan analitikal gel kromatografi atas kolum Sephacryl S-200 HR yang telah terkalibrat untuk menentukan jejari Stokes. C₈₁ BLA didapati mempunyai jejari Stokes yang paling tinggi diikuti dengan S₄₂ dan G₈₈ BLA. Kestabilan konformasi untuk protein asli dan derivative modifikasi disiasat dengan denaturasi urea menggunakan 'circular dichroism' (CD) spektroskopi. Profil denaturasi protein-protein tersebut menunjukkan pengurangan kestabilan dalam protein yang telah termodifikasikan terutamanya dalam penyediaan C₈₁ BLA. Selain itu, impak kalsium atas urea dan GdnHCl denaturasi protein asli dan BLA di mana intrinsik kalsium yang telah dikeluarkan ('Ca-depleted BLA') juga dianalisa dengan menggunakan CD, 'fluorescence' spektroskopi dan aktiviti biologi. Berdasarkan keputusan tersebut, kalsium ditemui sebagai ejen penstabil BLA daripada urea dan GdnHCl denaturasi.

ACKNOWLEDGEMENTS

First and foremost, I offer my most sincere gratitude to my supervisor, **Professor Dr. Saad Tayyab** who has supported me throughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I attribute the level of my Master degree to his encouragement and effort. Without his guidance, this thesis would not have been completed or written. One simply could not wish for a better supervisor.

I also wish to express my gratitude to my co-supervisor, **Associate Professor Dr. Habsah Abdul Kadir** who was abundantly helpful in providing moral support and all necessary facilities needed for the completion of this project. I too, wish to thank **Professor Raja Noor Zaliha binti Raja Abdul Rahman** for allowing me to use the CD machine in her laboratory at Universiti Putra Malaysia.

Deepest gratitude is also due to **Professor Dr. Rosli Hashim**, Head, Institute of Biological Sciences and **Professor Dato' Dr. Mohd. Sofian Azirun**, Dean, Faculty of Science, University of Malaya for providing a good working atmosphere.

In my daily work, I have been blessed with a friendly and cheerful group of colleagues, Bavani Arumugam, Adyani Azizah, Nabilah Sidek and Faizah Mohd Faizul who were always offering their support when in need. Special thanks go to Wong Chee Fah for helping me with the usage of CD machine in Universiti Putra Malaysia.

My mother, brother and Stze Woi, thank you all for the encouragement, inspiration, understanding and endless love throughout this project.

I would also like to convey thanks to the Ministry of Higher Education, Malaysia for the initial support in the form of Research Assistantship of the Fundamental Research Grant Scheme (FP081/2007C) sanctioned to my supervisor. Financial support in the form of University Malaya Fellowship and IPPP grant are also greatly acknowledged.

I would like to acknowledge the publishers, Springer Science, New York and Elsevier, Oxford for permitting the usage of figures and tables in this dissertation.

(TAN CHEAU YUAAN)

Date:

TABLE OF CONTENTS

	Page
ABSTRACT	iii
ABSTRAK	iv
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vii
LIST OF FIGURES	xi
LIST OF TABLES	xiv
LIST OF ABBREVIATIONS	xv
CHAPTER 1 INTRODUCTION	1
CHAPTER 2 LITERATURE REVIEW	4
2.1 Background	4
2.2 Isolation and purification	5
2.3 Physicochemical properties	11
2.4 Structural organization	13
2.4.1 Amino acid composition	13
2.4.2 Primary structure	13
2.4.3 Three-dimensional structure	13
2.4.4 Active site, metal binding sites and ionic interactions	21
2.5 Stability	26
2.5.1 pH	26
2.5.2 Temperature	29
2.5.3 Surfactants / Detergents	31
2.5.4 Chemical denaturants	31
CHAPTER 3 MATERIALS AND METHODS	34

3.1	Materials	34
3.1.1	Proteins	34
3.1.2	Reagents used in protein estimation	34
3.1.3	Reagents used in enzyme assay	34
3.1.4	Reagents used in chemical modification	34
3.1.5	Reagents used in polyacrylamide gel electrophoresis	35
3.1.6	Reagents used in gel chromatography	35
3.1.7	Reagents used in denaturation experiments	35
3.1.8	Miscellaneous	35
3.2	Methods	36
3.2.1	pH measurements	36
3.2.2	Absorption spectroscopy	36
3.2.3	Fluorescence spectroscopy	37
3.2.4	Circular dichroism spectroscopy	37
3.2.5	Preparation of calcium-depleted BLA	38
3.2.6	Determination of protein concentration	38
3.2.6.1	Spectrophotometric method	38
3.2.6.2	Method of Lowry <i>et al.</i> (1951)	38
3.2.7	Analytical methods	39
3.2.8	Enzyme assay	41
3.2.9	Chemical modifications of BLA	43
3.2.9.1	Succinylation	43
3.2.9.2	Carbamylation	44
3.2.9.3	Guanidination	44
3.2.10	Quantification of modification	44
3.2.11	Polyacrylamide gel electrophoresis	45

3.2.12	Analytical gel chromatography	47
3.2.13	Denaturation experiments	49
3.2.13.1	GdnHCl denaturation of native and Ca-depleted BLAs	49
3.2.13.2	Urea denaturation of native, Ca-depleted and modified BLAs	50
3.2.13.3	Data analysis	50
CHAPTER 4	RESULTS	52
4.1	Effect of calcium on GdnHCl denaturation of native and Ca-depleted BLAs	52
4.1.1	Circular dichroism	52
4.1.2	Intrinsic fluorescence	55
4.1.3	Enzymatic activity	61
4.2	Chemical modifications of lysine residues of BLA	61
4.3	Conformational changes in modified BLAs	66
4.4	Conformational stabilities of native, Ca-depleted and modified BLAs	71
CHAPTER 5	DISCUSSION	89
5.1	Effect of calcium on GdnHCl denaturation of native and Ca-depleted BLAs	89
5.1.1	Circular dichroism	89
5.1.2	Intrinsic fluorescence	91
5.1.3	Enzymatic activity	92
5.2	Chemical modifications of lysine residues of BLA	94
5.3	Conformational changes in modified BLAs	95
5.4	Conformational stabilities of native, Ca-depleted and modified BLAs	97
CHAPTER 6	CONCLUSION	102

REFERENCES	103
LIST OF PUBLICATIONS / PRESENTATIONS	113
BIOGRAPHY	118

LIST OF FIGURES

	Page
Figure 2.1: Primary structure of BLA.	15
Figure 2.2: Three-dimensional structures of various α -amylases.	16
Figure 2.3: A representation of all β structures in domain B of BLA.	20
Figure 2.4: Tertiary structure of <i>Bacillus licheniformis</i> α -amylase.	25
Figure 3.1: Standard curves for the determination of protein concentration by the method of Lowry <i>et al.</i> (1951) using bovine serum albumin as the standard.	40
Figure 3.2: Standard curve for the determination of maltose concentration, produced in the activity assay of BLA by the method of Bernfeld (1951).	42
Figure 4.1: GdnHCl denaturation of native BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence and presence of 2 mM CaCl_2 as followed by MRE measurements at 222 nm.	53
Figure 4.2: GdnHCl denaturation of Ca-depleted BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence and presence of 2 mM CaCl_2 as followed by MRE measurements at 222 nm.	54
Figure 4.3: GdnHCl denaturation of native BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence and presence of 2 mM CaCl_2 as followed by fluorescence measurements at 336 nm upon excitation at 280 nm.	56
Figure 4.4: GdnHCl denaturation of Ca-depleted BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence and presence of 2 mM CaCl_2 as followed by fluorescence measurements at 338 nm upon excitation at 280 nm.	58
Figure 4.5: GdnHCl denaturation of native BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence and presence of 2 mM CaCl_2 as followed by change in emission maximum upon excitation at 280 nm.	59
Figure 4.6: GdnHCl denaturation of Ca-depleted BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence and presence of 2 mM CaCl_2 as followed by change in emission maximum upon excitation at 280 nm.	60

Figure 4.7:	Effect of increasing GdnHCl concentrations on the specific activity of native BLA in the absence and presence of 2 mM CaCl ₂ .	62
Figure 4.8:	Effect of increasing GdnHCl concentrations on the specific activity of Ca-depleted BLA in the absence and presence of 2 mM CaCl ₂ .	63
Figure 4.9:	TNBSA color reaction to determine the extent of modification in succinylated, carbamylated and guanidinated BLA preparations.	64
Figure 4.10:	PAGE pattern of native (N), 42% succinylated (S ₄₂), 81% carbamylated (C ₈₁) and 88% guanidinated (G ₈₈) BLA preparations on 10% polyacrylamide gel performed according to Laemmli (1970) under non-denaturing condition.	67
Figure 4.11:	Elution profiles of blue dextran, BSA monomer, carbonic anhydrase, native BLA and cytochrome c on Sephacryl S-200 HR column (1.63×56 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.5 containing 0.02% sodium azide.	68
Figure 4.12:	Elution profiles of α-chymotrypsinogen A and G ₈₈ BLA on Sephacryl S-200 HR column (1.63×56 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.5 containing 0.02% sodium azide.	69
Figure 4.13:	Elution profiles of C ₈₁ BLA, S ₄₂ BLA and L-tyrosine on Sephacryl S-200 HR column (1.63×56 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.5 containing 0.02% sodium azide.	70
Figure 4.14:	Stokes' radii determination of native, S ₄₂ , C ₈₁ and G ₈₈ BLAs according to Ackers (1967).	73
Figure 4.15:	Stokes' radii determination of native, S ₄₂ , C ₈₁ and G ₈₈ BLAs according to Laurent and Killander (1964).	74
Figure 4.16:	Plots of relative MRE _{222 nm} of native BLA in 0.02 M Tris-HCl buffer, pH 7.5 at 25°C versus urea concentration, in the absence and presence of 2 mM CaCl ₂ .	76
Figure 4.17:	Plots of relative MRE _{222 nm} of Ca-depleted BLA in 0.02 M Tris-HCl buffer, pH 7.5 at 25°C versus urea concentration in the absence and presence of 2 mM CaCl ₂ .	77
Figure 4.18:	Plot of relative MRE _{222 nm} of S ₄₂ BLA in 0.02 M Tris-HCl buffer, pH 7.5 at 25°C versus urea concentration.	80

Figure 4.19:	Plot of relative $MRE_{222\text{ nm}}$ of C ₈₁ BLA in 0.02 M Tris-HCl buffer, pH 7.5 at 25°C versus urea concentration.	81
Figure 4.20:	Plot of relative $MRE_{222\text{ nm}}$ of G ₈₈ BLA in 0.02 M Tris-HCl buffer, pH 7.5 at 25°C versus urea concentration.	82
Figure 4.21:	(A) Normalized transition curve for urea denaturation of native BLA in terms of F_D versus urea concentration. (B) Plot of ΔG_D against urea concentration.	84
Figure 4.22:	(A) Normalized transition curve for urea denaturation of Ca-depleted BLA in terms of F_D versus urea concentration. (B) Plot of ΔG_D against urea concentration.	85
Figure 4.23:	(A) Normalized transition curve for urea denaturation of S ₄₂ BLA in terms of F_D versus urea concentration. (B) Plot of ΔG_D against urea concentration.	86
Figure 4.24:	(A) Normalized transition curve for urea denaturation of C ₈₁ BLA in terms of F_D versus urea concentration. (B) Plot of ΔG_D against urea concentration.	87
Figure 4.25:	(A) Normalized transition curve for urea denaturation of G ₈₈ BLA in terms of F_D versus urea concentration. (B) Plot of ΔG_D against urea concentration.	88

LIST OF TABLES

	Page
Table 1.1: Properties of several α -amylases from <i>Bacillus sp.</i>	2
Table 2.1: α -Amylase producing microorganisms.	6
Table 2.2: Purification strategies employed for different α -amylases.	9
Table 2.3: Physicochemical properties of BLA.	12
Table 2.4: Amino acid composition of <i>Bacillus licheniformis</i> α -amylase.	14
Table 2.5: Secondary structure elements of <i>Bacillus licheniformis</i> α -amylase as assigned with DSSP.	18
Table 2.6: Residues involved in the active site of different α -amylases.	22
Table 2.7: Distances between the metal ions in BLA and their ligands.	24
Table 2.8: Ionic interactions in BLA.	27
Table 2.9: Stable pH ranges of some α -amylases.	28
Table 4.1: Chemical modifications of amino groups of BLA.	65
Table 4.2: Analytical gel chromatographic data of marker proteins, native and modified BLAs on Sephacryl S-200 HR column (1.63 \times 56 cm) at pH 7.5.	72
Table 4.3: Stokes radii of native, 42% succinylated, 81% carbamylated and 88% guanidinated BLAs at pH 7.5.	75
Table 4.4: Urea denaturation data of native, Ca-depleted, 42% succinylated, 81% carbamylated and 88% guanidinated BLAs at pH 7.5 as monitored by MRE measurements at 222 nm.	79

LIST OF ABBREVIATIONS

ANS	8-Anilino-1-napthalene sulfonic acid
BAA	<i>Bacillus amyloliquefaciens</i> α -amylase
BLA	<i>Bacillus licheniformis</i> α -amylase
BSA	Bovine serum albumin
BStA	<i>Bacillus stearothermophilus</i> α -amylase
BSUA	<i>Bacillus subtilis</i> α -amylase
<i>c</i>	Protein concentration in mg/ml
$^{\circ}\text{C}$	Degree Celsius
C ₈₁	81% Carbamylated
Ca	Calcium
CaCl ₂	Calcium chloride
cal/mol/M	Calories/mole/molar
CD	Circular dichroism
cm	Centimeter
[D]	Denaturant concentration
deg.	Degree
dmol	Decimole
DMSO	Dimethyl sulfoxide
DNS	Dinitrosalicylic acid
<i>e.g.</i>	Latin phrase <i>exempli gratia</i> (for example)
EGTA	Ethylene glycol-bis (2-aminoethyl-ether)-N,N,N',N'-tetraacetic acid
etc	Latin phrase <i>et cetera</i> (and so on)
F _D	Fraction denatured
g	Gram
G ₈₈	88% Guanidinated
ΔG_D	Free energy change
$\Delta G_D^{\text{H}_2\text{O}}$	Free energy of stabilization
GdnHCl	Guanidine hydrochloride
Glu	Glutamic acid
h	Hour
<i>h</i>	Height
HCl	Hydrochloric acid
<i>i.e.</i>	Latin phrase <i>id est</i> (that is)
K _{av}	Available distribution coefficient
K _D	Equilibrium constant
K _d	Distribution coefficient
<i>l</i>	optical path length of the cell
Lys	Lysine
M	Molar
m	Slope value
<i>m</i>	Measure of the dependence of ΔG_D on denaturant concentration
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
MRE	Mean residue ellipticity
MRE _{222 nm}	Mean residue ellipticity at 222 nm
MRW	Mean residue weight

NaCl	Sodium chloride
NaOH	Sodium hydroxide
No.	Number
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
Phe	Phenylalanine
R	Gas constant
r	Radius
S ₄₂	42% Succinylated
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SmF	Submerged fermentation
SSF	Solid-state fermentation
T	Absolute temperature
TEMED	N, N, N', N'-Tetramethylethylenediamine
T _m	Melting temperature
TNBSA	Trinitrobenzenesulfonic acid
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloride
Trp	Tryptophan
Tyr	Tyrosine
μg	Microgram
μM	Micromolar
UV	Ultraviolet
V	Volume
V	Voltage
V _i	Inner volume
V _o	Void volume
V _t	Total volume
v/v	Volume per volume
w/v	Weight per volume
Y	Variable parameter
Y _D	Variable parameter for the denatured state
Y _N	Variable parameter for the native state
θ _{obs}	Observed ellipticity
~	Approximate
≥	Greater than or equal to
%	Percentage
±	Plus-minus